

## DESCRIPTION

### METHOD FOR MEASUREMENT USING SULFONIC ACID COMPOUND AND NITRO COMPOUND

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#### Technical Field

The present invention relates to a method for measuring an analyte in a sample by using a redox reaction.

#### 10 Background Art

Conventionally, the measurement of the amount of an analyte in a sample using a redox reaction has been utilized for a wide range of applications. For example, such measurement has been utilized for measuring glycated proteins in applications such as biochemical analyses, clinical tests, and the like.

For instance, glycated proteins in blood, particularly glycated hemoglobin in erythrocytes, are significant indicators in the diagnosis, therapy and the like of diabetes because they reflect the patient's past history of blood glucose levels. Such glycated proteins in erythrocytes are measured using the above-noted redox reaction, for example, in the following manner.

First, erythrocytes are hemolyzed to prepare a sample. This hemolyzed sample is treated with a fructosyl amino acid oxidase (hereinafter, referred to as "FAOD") so that the FAOD is allowed to act on a glycated portion of the glycated protein, thus generating hydrogen peroxide. The amount of this hydrogen peroxide formed corresponds to the amount of the glycated protein. Then, a peroxidase (hereinafter, referred to as "POD") and a substrate that develops color by oxidation (a chromogenic substrate) further are added to this sample, so that a redox reaction occurs between the hydrogen peroxide and the chromogenic substrate with the POD as a catalyst. At this time, since the oxidizing substrate develops color when it is oxidized, the amount of the hydrogen peroxide can be determined by measuring the color developed. As a result, the amount of the glycated protein in erythrocytes can be determined.

However, various kinds of reducing substances such as ascorbic acid (AsA) and bilirubin usually are present in blood. Moreover, various kinds of reducing substances such as glutathione (GSH) are present in erythrocytes. These reducing substances may, for example, reduce the hydrogen peroxide,

may inhibit the redox reaction, or may reduce the reducing agent after it develops color, which causes degradation of the color. Therefore, there has been a problem that it is difficult to determine the amount of the glycated protein in erythrocytes accurately.

5 Also, there has been another problem that the accuracy of the determination may deteriorate because the concentration of the contained reducing substances is not constant in each sample.

In order to avoid these problems, for example, various kinds of oxidizing agents have been added to samples. For example, JP  
10 56(1981)–151358 A discloses a method of using halogen oxides such as iodic acid or periodic acid as oxidizing agents. JP 57(1982)–13357 A, JP 57(1982)–161650 A, JP 59(1984)–193354 A, JP 62(1987)–169053 A and JP 3(1991)–30697 A also disclose methods of using complexes of metals such as cobalt, iron, cerium, etc. as oxidizing agents.

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#### Disclosure of Invention

However, in these conventional methods, the accuracy of determination sometimes does not improve sufficiently depending on the samples. Also, as mentioned earlier, since glycated proteins in blood are  
20 significant indicators in the diagnosis, therapy and the like of diabetes, it has been desired that the method for measuring them by using a redox reaction achieve a still higher accuracy of determination.

Accordingly, it is an object of the present invention to provide a highly reliable method for measuring an analyte in a sample by using a redox  
25 reaction.

In order to achieve the above-mentioned objects, a measuring method according to the present invention is a method for measuring an analyte in a sample containing hemoglobin or a hemoglobin degradation product by using a redox reaction, including adding at least one of a sulfonic acid compound  
30 and a nitro compound to the sample so as to eliminate an influence of the hemoglobin or the hemoglobin degradation product contained in the sample.

The inventors of the present invention found that (i) the conventional methods described above merely eliminate the influence of low-molecular-weight reducing substances such as the AsA and GSH but do  
35 not eliminate that of high-molecular-weight reducing substances such as proteins and the like and further (ii) the treatment of the sample with a tetrazolium compound eliminates not only the influence of the

low-molecular-weight reducing substances but also the influence of the high-molecular-weight reducing substances, especially that of hemoglobin and a hemoglobin degradation product (hereinafter, both of them are altogether referred to as "hemoglobin") as a redox substance. With regard to these findings, the applicant filed another application. However, because of its low solubility, it is difficult to make the above-noted tetrazolium compound correspond to hemoglobin contained in samples with a high concentration. Since the tetrazolium compound itself is oxidative, there has been a problem that it may act on the substrate that develops color by oxidation, thus developing erroneous color. In this context, the inventors of the present invention conducted a keen study with a view to eliminating the influence of hemoglobin without affecting the measurement system. As a result, the inventors found that, by treating the sample with a sulfonic acid compound and a nitro compound, the influence of hemoglobin was prevented without affecting the measurement system, thus arriving at the present invention. With such a measuring method of the present invention, it becomes possible to conduct a still more accurate determination, which is useful for various tests in clinical medicine as described above.

#### 20 Best Mode for Carrying Out the Invention

As described above, the measuring method according to the present invention is a method for measuring an analyte in a sample by using a redox reaction, including adding at least one of a sulfonic acid compound and a nitro compound to the sample, thereby eliminating an influence of the hemoglobin contained in the sample on a measurement system.

More specifically, it is preferable that, prior to the redox reaction, at least one of the sulfonic acid compound and the nitro compound is added to the sample so as to eliminate the influence of the hemoglobin or the hemoglobin degradation product contained in the sample and thereafter, the method further includes forming an oxidizing substance or a reducing substance derived from the analyte, measuring the amount of the formed substance derived from the analyte by the redox reaction, and determining the amount of the analyte from the measurement value indicating the amount of the formed substance.

35 In the present invention, the substance to be added for eliminating the influence of hemoglobin may be either one of the sulfonic acid compound and the nitro compound, for example. However, it is preferable that both of

these compounds are added to the sample because the influence can be eliminated even further. When both of the sulfonic acid compound and the nitro compound are added, they need not be added in any particular order and may be added at the same time or at separate times.

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In the present invention, the sulfonic acid compound can be a compound represented by, for example, a general formula:  $R-SO_3X$ .

10 In the above formula, X is, for example, Na, K, Li, H or the like, and R preferably is a hydrophobic group, for example,  $CH_3(CH_2)_n-$ ,  $CH_3(CH_2)_n-C_6H_4-$ ,  $C_6H_5-$ ,  $C_6H_5-N=N-C_6H_4-$ ,  $C_6H_5-CH=CH-C_6H_4-$  or the like. For example, n in the above R ranges from 1 to 20. In the above R, "H" may be substituted by an acyl group, a nitro group, a nitroso group, a phenyl group, an alkyl group, and an alkyl ether group or the like.

15 Specific examples of the sulfonic acid compound include, for example, sodium lauryl sulfate (hereinafter, referred to as "SLS"), dodecylbenzenesulfonic acid sodium salt (hereinafter, referred to as "SDBS"), lithium lauryl sulfate (hereinafter, referred to as "LiLS"), 4-aminoazobenzene-4'-sulfonic acid sodium salt (hereinafter, referred to as "ABSA"), 4-amino-4'-nitrostilbene-2,2'-disulfonic acid disodium salt  
20 (hereinafter, referred to as "ANDS"), 4,4'-diazidostilbene-2,2'-disulfonic acid disodium salt (hereinafter, referred to as "DADS"), N-cyclohexyl-2-aminoethane sulfonic acid, N-cyclohexyl-3-aminopropane sulfonic acid, N-cyclohexyl-2-hydroxy-3-aminopropane sulfonic acid,  
25 piperazine-1,4-bis(2-ethane sulfonic acid), bathophenanthroline sulfonic acid and the like, and the sulfonic acid compound more preferably is SLS, SDBS or LiLS.

30 In the present invention, the nitro compound is not particularly limited but can be, for example, a nitrobenzene compound or a dinitrobenzene compound. A benzene ring of these compounds preferably has not only the nitro group but also a substituent such as  $-NH_2$ ,  $-OH$ ,  $-COOH$ ,  $-SO_3$  or  $-(CH_2)_nCH_3$  (n = 2 to 9) and, among them, preferably has a hydrophilic group as the substituent. The substituent can be, for example, a halogen group, an  
35 ether group or a phenyl group.

Specific examples of the nitro compound include, for example, 2,4-dinitrophenol (2,4-DNP), 2,5-dinitrophenyl, 2,6-dinitrophenyl,

4,6-dinitro-2-methyl phenol, 2-amino-4-nitrophenol, 2-amino-5-nitrophenol, p-nitrophenol (p-NP), 2,4-dinitroaniline (2,4-DNA), p-nitroaniline (p-NA), sodium nitrite ( $\text{NaNO}_2$ ), potassium nitrite ( $\text{KNO}_2$ ), 4-amino-4'-nitrostilbene-2,2'-disulfonic acid disodium salt (hereinafter, referred to as "ANPS"), nitrobenzene and the like.

When using both the sulfonic acid compound and the nitro compound, there is no particular limitation on the combination thereof.

Since these sulfonic acid compound and nitro compound have a higher solubility than, for example, the tetrazolium compound mentioned above, they can be treated easily even when the concentration of hemoglobin in the sample is high. Also, in view of their inexpensiveness, the sulfonic acid compound and nitro compound are very useful.

In the measuring method according to the present invention, the amount of the sulfonic acid compound and nitro compound to be added is not particularly limited but can be determined suitably depending on the kind of sample, the amount of hemoglobin contained in the sample, the amount of other reducing substances, etc. Specific examples follow.

In the case of adding either the sulfonic acid compound or the nitro compound, the compound is added preferably in the range of 0.01 to 1000  $\mu\text{mol}$ , more preferably in the range of 0.03 to 200  $\mu\text{mol}$  and particularly preferably in the range of 0.05 to 40  $\mu\text{mol}$  with respect to 1  $\mu\text{l}$  of the sample.

In the case of adding both the sulfonic acid compound and the nitro compound, the sulfonic acid compound is added preferably in the range of 0.005 to 20  $\mu\text{mol}$  and the nitro compound is added preferably in the range of 0.005 to 25  $\mu\text{mol}$ , and the former is added more preferably in the range of 0.02 to 4  $\mu\text{mol}$  and the latter is added more preferably in the range of 0.01 to 5  $\mu\text{mol}$  with respect to 1  $\mu\text{l}$  of the sample.

In the present invention, it is preferable that the redox reaction is a color development reaction caused by reducing the oxidizing substance derived from the analyte and oxidizing a substrate that develops color by oxidation (a chromogenic substrate) using an oxidase, and the amount of the oxidizing substance is measured by measuring a degree of the color developed in the color development reaction. The degree of the color developed is measured by, for example, measuring an absorbance at a wavelength for

detecting the substrate.

The chromogenic substrate can be, for example,  
N-(carboxymethylaminocarbonyl)-4,4'-bis(dimethylamino)diphenylamine  
sodium salt (hereinafter, referred to as "DA-64"), a combination of Trinder's  
5 reagent and 4-aminoantipyrine,  
N,N,N',N',N'',N''-hexa(3-sulfopropyl)-4,4',4''-triaminotriphenylmethane  
hexasodium salt (hereinafter, referred to as "TPM-PS"),  
N,N,N',N',N'',N''-hexa(2-hydroxy-3-sulfopropyl)-4,4',4''-triaminotriphenylmeth  
ane hexasodium salt (hereinafter, referred to as "TPM-OS"),  
10 10-(carboxymethylaminocarbonyl)3,7-bis(dimethylamino) phenothiazine  
sodium salt (hereinafter, referred to as "DA-67"),  
10-(methylaminocarbonyl)3,7-bis(dimethylamino) phenothiazine (hereinafter,  
referred to as "MCDP"),  
10-(carboxyaminomethyl-4-benzaminocarbonyl)3,7-bis(dimethylamino)  
15 phenothiazine sodium salt (hereinafter, referred to as "MMX") or the like.  
Among the above, triphenylmethane-based chromogenic substrates such as  
TPM-PS and TPM-OS are preferable, and in particular,  
triaminotriphenylmethane-based chromogenic substrates are preferable.

The Trinder's reagent can be, for example, phenols, phenol  
20 derivatives, aniline derivatives, naphthols, naphthol derivatives,  
naphthylamine or naphthylamine derivatives. The compound to be  
combined with the Trinder's reagent may be not only 4-aminoantipyrine  
noted above but also, for example, aminoantipyrine derivatives, vanillin  
diamine sulfonic acid, methyl benzothiazolinone hydrazone (MBTH),  
25 sulfonated methyl benzothiazolinone hydrazone (SMBTH) or the like.

The chromogenic substrates illustrated above usually have an  
absorbance at 400 nm or longer. Thus, in the case of using the  
above-described tetrazolium compound also having an absorbance at 400 nm  
or longer, the presence of the tetrazolium compound may cause error in the  
30 measurement value. On the other hand, the sulfonic acid compound and the  
nitro compound illustrated above do not have an absorbance at 400 nm or  
longer, so that there is no need to worry about the error caused in the  
absorbance measurement even when used with these chromogenic  
substrates.

35 Although there is no particular limitation on the combination of these  
chromogenic substrates with the sulfonic acid compound and the nitro

compound, the following combinations are preferable for achieving an accurate color development reaction, for example. Incidentally, the reason that the accuracy of color development reaction improves according to the combination of the chromogenic substrates with the sulfonic acid compound and the nitro compound is not known.

In the case where the chromogenic substrate is DA-64 or a combination of the Trinder's reagent and 4-aminoantipyrine, it is preferable to add both of the sulfonic acid compound and the nitro compound to the sample. For example, the sulfonic acid compound preferably is SLS, SDBS or LiLS, and the nitro compound preferably is 2,4-DNA, 2,4-DNP, PNA or PNP.

On the other hand, in the case where the chromogenic substrate is TPM-PS, TPM-OS, DA-67, MCDP or MMX, it is preferable to add the sulfonic acid compound to the reagent, and it is more preferable to add both of the sulfonic acid compound and the nitro compound to the reagent.

In the present invention, it is preferable that the oxidizing substance derived from the analyte is hydrogen peroxide. It is also preferable that the oxidase is a peroxidase.

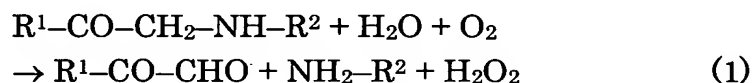
In the measuring method of the present invention, the kind of the sample is not particularly limited. Other than blood samples such as whole blood, plasma, serum and blood cells, the samples can be, for example, biological samples such as urine, spinal fluid and saliva, drinks such as juices, or foods such as soy sauce and Worcestershire sauce.

In the measuring method of the present invention, the analyte is not particularly limited as long as it utilizes a redox reaction but may be, for example, components in whole blood, components in erythrocytes, components in plasma, components in serum and the like, and it preferably is a component in erythrocytes. More specifically, the analyte may be, for example, glycated proteins such as glycated hemoglobin and glycated albumin, glycated peptides, glycated amino acids, glucose, uric acid, cholesterol, creatinine, sarcosine, glycerol and the like. Among these, glycated proteins are more preferable. For example, when a component in erythrocytes is to be measured, whole blood itself may be hemolyzed to prepare a sample, or erythrocytes are separated from whole blood and

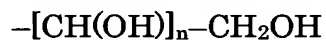
hemolyzed to prepare a sample.

In the measuring method of the present invention, when the analyte is a glycated protein, it is preferable that a FAOD is caused to act on the glycated protein so that hydrogen peroxide is formed as the oxidizing substance derived from the analyte. The glycated portion of the glycated protein is oxidized and decomposed by the FAOD, thereby generating the hydrogen peroxide. Also, when the analyte is a glycated amine such as a glycated peptide or a glycated amino acid, it is preferable that the analyte similarly is subjected to the action of a FAOD. Moreover, it is preferable that glycated proteins and glycated peptides are treated with a protease before the FAOD treatment as necessary.

As the FAOD, a FAOD catalyzing a reaction represented by Formula (1) below preferably is used.



In Formula (1),  $R^1$  represents a hydroxyl group or a residue derived from the sugar before glycation (i.e., sugar residue). The sugar residue ( $R^1$ ) is an aldose residue when the sugar before glycation is aldose, and is a ketose residue when the sugar before glycation is ketose. For example, when the sugar before glycation is glucose, it takes a fructose structure after glycation by an Amadori rearrangement. In this case, the sugar residue ( $R^1$ ) becomes a glucose residue (an aldose residue). This sugar residue ( $R^1$ ) can be represented, for example, by



where  $n$  is an integer of 0 to 6.

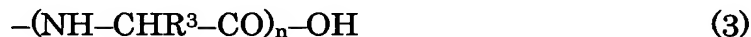
In Formula (1),  $R^2$  is not particularly limited. However, when the substrate is a glycated amino acid, a glycated peptide or a glycated protein, for example, there is a difference between the case where an  $\alpha$ -amino group is glycated and the case where an amino group other than the  $\alpha$ -amino group is glycated.

In Formula (1), when an  $\alpha$ -amino group is glycated,  $R^2$  is an amino acid residue or a peptide residue represented by Formula (2) below.

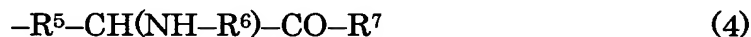




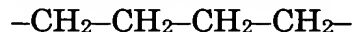
In Formula (2), R<sup>3</sup> denotes an amino-acid side chain group. R<sup>4</sup> denotes a hydroxyl group, an amino acid residue or a peptide residue, and can be represented, for example, by Formula (3) below. In Formula (3), n is an integer of 0 or larger, and R<sup>3</sup> denotes an amino-acid side chain group as in the above.



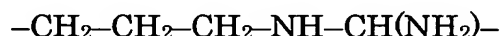
In Formula (1) above, when an amino group other than the α-amino group is glyated (i.e., an amino-acid side chain group is glyated), R<sup>2</sup> can be represented by Formula (4) below.



In Formula (4) above, R<sup>5</sup> denotes a portion other than the glyated amino group in the amino-acid side chain group. For example, when the glyated amino acid is lysine, R<sup>5</sup> is as follows.



For another example, when the glyated amino acid is arginine, R<sup>5</sup> is as follows.

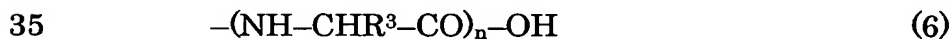


In Formula (4) above, R<sup>6</sup> denotes hydrogen, an amino acid residue or a peptide residue, and can be represented, for example, by Formula (5) below.

In Formula (5), n denotes an integer of 0 or larger, and R<sup>3</sup> denotes an amino-acid side chain group as in the above.



In Formula (4) above, R<sup>7</sup> denotes a hydroxyl group, an amino acid residue or a peptide residue, and can be represented, for example, by Formula (6) below. In Formula (6), n is an integer of 0 or larger, and R<sup>3</sup> denotes an amino-acid side chain group as in the above.



(First Embodiment)

In the following, the measuring method of the present invention will be described with reference to the examples in which a glycated protein in blood cells is measured.

First, whole blood itself is hemolyzed, or a blood cell fraction is  
5 separated from whole blood in the usual way such as centrifugation and then hemolyzed, so as to prepare a hemolyzed sample. The method of causing the hemolysis is not particularly limited and can be, for example, a method using a surfactant, a method using ultrasonic waves, a method utilizing a difference in osmotic pressure or the like. Among these, the method using a surfactant  
10 is preferable because of its simplicity in operation, etc.

As the surfactant, for example, nonionic surfactants such as polyoxyethylene-p-t-octylphenyl ether (e.g. Triton series surfactants), polyoxyethylene sorbitan alkyl ester (e.g. Tween series surfactants), polyoxyethylene alkyl ether (e.g. Brij series surfactants) and the like can be  
15 used. Specific examples thereof are Triton X-100, Tween-20, Brij 35, etc. The conditions of the treatment with the surfactant usually are as follows: when the concentration of blood cells in the solution to be treated is 1 to 10 vol%, the surfactant is added so that its concentration in the solution falls in the range from 0.01 to 5 wt%, and stirred at room temperature for about  
20 several seconds (about 5 seconds) to 10 minutes.

Next, at least one of a sulfonic acid compound and a nitro compound is added to the hemolyzed sample to carry out pretreatment of the hemolyzed sample.

25 Although either one of or both of the sulfonic acid compound and the nitro compound may be added, it is preferable to make decisions suitably according to the kinds of chromogenic substrates to be used in a later step as described above.

In the case of adding either one of the sulfonic acid compound and the  
30 nitro compound, the amount to be added is not particularly limited but can be in the above-described addition ratio. More specifically, when the concentration of blood cells in the solution to be pretreated is 1 vol%, the concentration of the nitro compound ranges, for example, from 0.05 to 500 mmol/L and preferably from 0.2 to 100 mmol/L. Also, when the  
35 concentration of blood cells in the solution to be pretreated is 1 vol%, the concentration of the sulfonic acid compound ranges, for example, from 0.05 to 200 mmol/L and preferably from 0.2 to 40 mmol/L.

Also, in the case of adding both of the sulfonic acid compound and the nitro compound to the sample, the amount to be added is not particularly limited but can be in the above-described addition ratio. More specifically, when the concentration of blood cells in the solution to be pretreated is 1 vol%,  
5 the concentration of the sulfonic acid compound ranges, for example, from 0.05 to 200 mmol/L and that of the nitro compound ranges, for example, from 0.05 to 250 mmol/L, and the former ranges preferably from 0.2 to 40 mmol/L and the latter ranges preferably from 0.1 to 50 mmol/L. When both of the sulfonic acid compound and the nitro compound are added, they need not be  
10 added in any particular order as described above and may be added at the same time.

Although the sulfonic acid compound and the nitro compound may be used as they are, they preferably are dissolved in solvents and used as a sulfonic acid compound solution and a nitro compound solution in view of  
15 simplicity of operation and treatment efficiency. The concentration of each of these solutions can be determined suitably depending on the kinds or the like. The concentration of the sulfonic acid compound solution ranges, for example, from 5 to 1000 mmol/L and ranges preferably from 5 to 400 mmol/L, and that of the nitro compound solution ranges, for example, from 0.5 to 100 mmol/L  
20 and preferably from 1 to 50 mmol/L. As the solvent, it is possible to use distilled water, a physiological salt solution, buffers and the like, and the buffer can be those listed below. Incidentally, both of the above compounds may be an individual or a combination of two or more.

This pretreatment usually is carried out in a buffer. Examples of the  
25 buffer include amine buffers, phosphate buffers, borate buffers and Good's buffers such as MOPS buffer, CHES buffer, CAPS buffer, CAPSO buffer and the like. Examples of buffer agents contained in the amine buffers include glycine, ethylamine, diethylamine, methylamine, dimethylamine, trimethylamine, Tris(hydroxymethyl)aminomethane, triethanolamine,  
30 glycinamide, and the like.

The pH of the above buffers preferably ranges from 7 to 12, more preferably from 8 to 11, and particularly preferably from 8 to 10.

The conditions of this pretreatment are not specifically limited. However, the pretreatment usually is carried out at a temperature ranging  
35 from 10°C to 37°C and for a period of 10 seconds to 60 minutes.

In the case of using the above-described sodium lauryl sulfate or the like as the sulfonic acid compound to be used for this pretreatment, it is

possible to carry out the above-described hemolysis and this pretreatment at the same time by adding a single substance (i.e., sodium lauryl sulfate or the like) since sodium lauryl sulfate itself functions as the surfactant.

5           Next, the pretreated hemolyzed sample is treated with a protease. This treatment is performed so that the FAOD used in the subsequent treatment may act on the analyte more easily.

          The kind of the protease is not particularly limited, and for example, protease K, subtilisin, trypsin, aminopeptidase, metalloprotease and the like  
10       can be used. The conditions of the protease treatment are determined suitably depending on the kind of the protease used, the kind and concentration of the glycated protein as the analyte, etc.

          More specifically, when the pretreated hemolyzed sample is treated using protease K as the protease, usually, the concentration of the protease in  
15       the reaction solution ranges from 10 to 30,000 mg/L, the concentration of blood cells in the reaction solution ranges from 0.05 to 15 vol%, a reaction temperature ranges from 15°C to 37°C, a reaction period ranges from 1 minute to 24 hours, and a pH ranges from 6 to 12. This protease treatment usually is carried out in a buffer. As the buffer, it is possible to use a buffer  
20       similar to that in the pretreatment.

          Next, a degradation product obtained by the protease treatment further is treated with the FAOD. This FAOD treatment catalyzes the reaction shown by Formula (1) above.

25           It is preferable that the FAOD treatment is carried out in a buffer as in the above protease treatment. The conditions of the FAOD treatment are determined suitably depending on the kind of the FAOD used, the kind and concentration of the glycated protein as the analyte, etc.

          More specifically, the conditions are as follows: the concentration of  
30       the FAOD in the reaction solution ranges from 50 to 50,000 U/L, the concentration of blood cells in the reaction solution ranges from 0.01 to 1 vol%, a reaction temperature ranges from 15°C to 37°C, a reaction period ranges from 1 to 60 minutes, and a pH ranges from 6 to 9. Moreover, the kind of the buffer is not particularly limited, and for example, the buffers similar to those  
35       in the protease treatment can be used.

          Next, the hydrogen peroxide formed in the FAOD treatment is

measured by a redox reaction using a POD and the chromogenic substrate.

The redox reaction usually is carried out in a buffer. The conditions of the reaction are determined suitably depending on the concentration of the hydrogen peroxide formed, etc. The conditions are usually as follows: the  
5 concentration of the POD in the reaction solution ranges from 10 to 100,000 IU/L, the concentration of the chromogenic substrate ranges from 0.005 to 30 mmol/l, a reaction temperature ranges from 15°C to 37°C, a reaction period ranges from 0.1 to 30 minutes, and a pH ranges from 5 to 9. Moreover, the kind of the buffer is not particularly limited, and for example, the buffers  
10 similar to those in the protease treatment and the FAOD treatment can be used.

In the redox reaction, for example, when the chromogenic substrate is used, the amount of the hydrogen peroxide can be determined by measuring the degree of the color developed (i.e. absorbance) in the reaction solution  
15 with a spectrophotometer. Then, using this concentration of the hydrogen peroxide and a calibration curve or the like, for example, the amount of the glycated protein in the sample can be determined.

The amount of the hydrogen peroxide can be determined not only by  
20 the above-described enzymatic method using a POD or the like but also by an electrical method, for example.

In this method for measurement, the pretreatment step with the sulfonic acid compound and the nitro compound is not particularly limited as  
25 long as it is carried out before the redox reaction actually occurs as described above. However, because the hydrogen peroxide is formed after the FAOD treatment, it is preferable that the pretreatment step is performed before the FAOD treatment. Moreover, although each of the treating steps may be carried out separately as described above, some of the treating steps also may  
30 be performed simultaneously, for example, in the combinations as follows:

- 1: hemolysis treatment + pretreatment
- 2: hemolysis treatment + pretreatment + protease treatment
- 3: protease treatment + FAOD treatment
- 4: FAOD treatment + POD redox treatment
- 35 5: protease treatment + FAOD treatment + POD redox treatment

Furthermore, the order of adding the sulfonic acid compound and the nitro compound and the order of adding the FAOD, the POD and the

chromogenic substrate also are not particularly limited.

5 With this method, by contacting the sample with the sulfonic acid compound and the nitro compound, it is possible to eliminate not only the influence of low-molecular-weight reducing substances such as GSH, AsA, dithiothreitol, cysteine and N-acetyl-cysteine, but also the influence of hemoglobin and hemoglobin degradation products, in particular, as reducing substances. Consequently, a highly accurate determination can be performed without affecting the redox reaction and the absorbance measurement, for example.

15 Furthermore, in the pretreatment step in the measuring method of the present invention, for example, oxidizing agents and enzymes other than the sulfonic acid compound and the nitro compound further may be used in combination. Examples of the oxidizing agents include EDTA-Fe and halogen oxides such as sodium iodoacetate, iodic acid and periodic acid, and examples of the enzymes include ascorbic acid oxidase and bilirubin oxidase. The amount of such an oxidizing agent to be added ranges, for example, from 0.001 to 0.1 mg with respect to 1  $\mu$ l of the sample.

20 In the measuring method of the present invention, the analyte is not particularly limited as long as a redox reaction is utilized. Examples of the analyte other than the glycated protein include glycated peptides, glycated amino acids, glucose, cholesterol, uric acid, creatinine, sarcosine and glycerol, as described above.

25 When the amount of each of the above-described examples of the analyte is measured by forming hydrogen peroxide, it is appropriate to form the hydrogen peroxide, for example, by action of: a glucose oxidase on the glucose; a cholesterol oxidase on the cholesterol; a uricase on the uric acid; a sarcosine oxidase on the creatinine; a sarcosine oxidase on the sarcosine; or a glycerol oxidase on the glycerol; respectively. The amount of the hydrogen peroxide can be measured in the manner similar to that described above. Moreover, glycated peptides and glycated amino acids can be measured, for example, in the manner similar to the measurement of the glycated protein described above.

35 Furthermore, after the treatment of hemoglobin and hemoglobin

degradation products in a sample with the sulfonic acid compound and the nitro compound, in the case of forming a reducing substance derived from the analyte, measuring the amount of the reducing substance by a redox reaction and then determining the amount of the analyte from the measurement value,  
5 the measurement can be carried out, for example, in the following manner.

When the analyte is glucose, for example, a reducing substance such as NADH or NADPH is formed using glucose dehydrogenase in the presence of NAD<sup>+</sup>, NADP<sup>+</sup> or the like. Then, the NADH or NADPH as a reducing substance derived from the analyte is measured by a redox reaction, using,  
10 for example, diaphorase and a substrate that develops color by reduction. Then, as described above, the amount of the analyte in the sample can be determined using the concentration of the reducing substance derived from the analyte and a calibration curve or the like. Furthermore, for example, cholesterol dehydrogenase can be used when the analyte is cholesterol, and  
15 sarcosine dehydrogenase can be used when the analyte is sarcosine.

As the substrate that develops color by reduction, although not particularly limited, for example, the above-mentioned chromogenic tetrazolium compound, 2,6-dichlorophenolindophenol or the like can be used.

## 20 EXAMPLES

Hereinafter, examples will be described together with comparative examples.

### (Example 1)

25 In Example 1, using TPM-PS as a chromogenic substrate, a hemoglobin sample containing fructosyl valine (in the following, referred to as "FV") was treated with a sulfonic acid compound and a nitro compound, thus measuring the amount of FV. The sample and reagents used here and the method will be described below.

30

#### (Preparation of sample to be measured)

Lyophilized hemoglobin was dissolved in purified water, thus preparing a 50 g/L hemoglobin solution. On the other hand, FV was produced according to JP 2(1990)-69644 A and dissolved in purified water,  
35 thus preparing a 1 mM FV solution. Then, 37  $\mu$ l of the hemoglobin solution, 60  $\mu$ l of the FV solution and 203  $\mu$ l of purified water were mixed so as to prepare a sample to be measured.

(First reagent)

	Sulfonic acid compound	3.2 mM, 6.4 mM or 12.8 mM
	Surfactant (polyoxyethylene(9)dodecyl ether)	1.85 g/L
5	CHES-CHES · Na buffer (pH 9.4)	40 mM
	MOPS-MOPS · Na buffer (pH 9.4)	15 mM

As the sulfonic acid compound of the first reagent, SLS (manufactured by Nacalai Tesque, Inc.), SDBS (manufactured by Wako Pure Chemical Industries, Ltd.), ABSA (manufactured by Tokyo Kasei Kogyo Co., Ltd.), ANDS (manufactured by Tokyo Kasei Kogyo Co., Ltd.) and DADS (manufactured by Tokyo Kasei Kogyo Co., Ltd.) were used individually.

(Second reagent)

15	Nitro compound (2,4-DNA; manufactured by Wako Pure Chemical Industries, Ltd.)	0 mM or 6.4 mM
	Metalloprotease (manufactured by ARKRAY, INC.)	2.0 MU/L
	CaCl <sub>2</sub>	2.5 mM
	NaCl	50 mM
20	MOPS-MOPS · Na buffer (pH 6.5)	1.0 mM

(Third reagent)

	FAOD (manufactured by ARKRAY, INC.)	18 KU/L
	POD (manufactured by Kikkoman Corporation)	67 KU/L
25	CaCl <sub>2</sub>	2.5 mM
	TPM-PS (manufactured by DOJINDO LABORATORIES)	0.25 mM
	ADA buffer (pH 7.0)	300 mM

(Method)

After 8.26  $\mu$ L of the first reagent was added to 0.14  $\mu$ L of the sample to be measured, 75.6  $\mu$ L of the second reagent further was mixed therein and allowed to stand at 37°C for 5 minutes. Then, 18.9  $\mu$ L of the third reagent was blended into this mixture and incubated at 37°C so as to allow a color development reaction. The absorbance after 5 minutes was measured with trade name JCA-BM8 (manufactured by JEOL, Ltd.). The measurement wavelengths were set to 571 nm for the main wavelength and 805 nm for the sub-wavelength. On the other hand, in the Comparative Example, the



absorbance was measured similarly to Example 1 described above except that the sulfonic acid compound in the first reagent and the nitro compound in the second reagent were not added. When the first reagent and the second reagent were mixed into the samples, the amounts of the added sulfonic acid compound were 0.189  $\mu\text{mol}$ , 0.378  $\mu\text{mol}$  and 0.755  $\mu\text{mol}$  and the amount of the added nitro compound was 3.46  $\mu\text{mol}$  with respect to 1  $\mu\text{L}$  of the sample.

(Table 1)

	Sulfonic acid compound (mM)	Nitro compound (mM)	Absorbance (mAbs)
Example 1-1	SLS (3.2)	2,4-DNA (0.9)	149
1-2	SLS (6.4)	2,4-DNA (0.9)	154
1-3	SLS (12.8)	2,4-DNA (0.9)	160
1-4	SDBS (6.4)	2,4-DNA (0.9)	121
1-5	SLS (6.4)	—	154
1-6	ABSA (6.4)	—	132
1-7	ANDS (6.4)	—	113
1-8	DADS (6.4)	—	124
Comparative Example 1	—	—	30

As shown in Table 1, in Example 1, with each of the sulfonic acid compounds or the combination thereof with the nitro compound, the absorbance indicating the FV amount in the samples increased compared with the Comparative Example. These results show that, according to Example 1, the sulfonic acid compound and the nitro compound eliminated the influence of hemoglobin in the samples.

#### (Example 2)

In Example 2, using DA-64 as a chromogenic substrate, a hemoglobin sample containing FV was treated with a sulfonic acid compound and a nitro compound, thus measuring the amount of FV. The sample and reagents used here and the method will be described below.

#### (Preparation of sample to be measured)

60  $\mu\text{L}$  of the hemoglobin solution prepared in Example 1 above, 37  $\mu\text{L}$  of the FV solution and 203  $\mu\text{L}$  of purified water were mixed so as to prepare a sample to be measured.

(First reagent)

	Sulfonic acid compound (SLS: manufactured by Nacalai Tesque, Inc.)	6.4 mM
	Surfactant (polyoxyethylene(9)lauryl ether)	1.85 g/L
	CHES-CHES · Na buffer (pH 9.4)	40 mM
5	MOPS-MOPS · Na buffer (pH 9.4)	15 mM

(Second reagent)

	Nitro compound	0.9 mM
	Metalloprotease (manufactured by ARKRAY, INC.)	2.0 MU/L
10	CaCl <sub>2</sub>	2.5 mM
	NaCl	50 mM
	MOPS-MOPS · Na buffer (pH 6.5)	1.0 mM

As the nitro compound, 2,4-DNA (manufactured by Wako Pure Chemical Industries, Ltd.), p-NA (manufactured by Wako Pure Chemical Industries, Ltd.), p-NP (manufactured by Wako Pure Chemical Industries, Ltd.), NaNO<sub>2</sub> (manufactured by Nacalai Tesque, Inc.), and 2,4-DNH (manufactured by Wako Pure Chemical Industries, Ltd.) were used individually. In the case of adding two kinds of the nitro compound, a total of 1.8 mM (0.9 mM each) was added.

(Third reagent)

	FAOD (manufactured by ARKRAY, INC.)	17.5 KU/L
	POD (manufactured by Kikkoman Corporation)	67 KU/L
25	DA-64 (manufactured by Wako Pure Chemical Industries, Ltd.)	70 μM
	Tris-HCL buffer (pH 7.0)	300 mM

(Method)

After 8.26 μL of the first reagent was added to 0.14 μL of the sample to be measured, 75.6 μL of the second reagent further was mixed therein and allowed to stand at 37°C for 5 minutes. Then, 18.9 μL of the third reagent was blended into this mixture and incubated at 37°C so as to allow a color development reaction. The absorbance after 5 minutes was measured with trade name JCA-BM8 (manufactured by JEOL, Ltd.). The measurement wavelengths were set to 751 nm for the main wavelength and 805 nm for the sub-wavelength. On the other hand, in the Comparative Example, the absorbance was measured similarly to Example 2 described above except that

the sulfonic acid compound in the first reagent and the nitro compound in the second reagent were not added. The results are shown in Table 2 below.

(Table 2)

		Sulfonic acid compound (mM)	Nitro compound (mM)	Absorbance (mAbs)
Example 2	2-1	SLS (6.4)	2,4-DNA (0.9)	51.3
	2-2	SLS (6.4)	p-NA (0.9)	41.0
	2-3	SLS (6.4)	p-NP (0.9)	41.5
	2-4	SLS (6.4)	NaNO <sub>2</sub> (0.9)	41.5
	2-5	SLS (6.4)	2,4-DNA (0.9)	277.0
			p-NP (0.9)	
	2-6	SLS (6.4)	2,4-DNA (0.9)	279.0
			p-NA (0.9)	
	2-7	SLS (6.4)	p-NP (0.9)	267.0
			p-NA (0.9)	
	2-8	SLS (6.4)	2,4-DNA (0.9)	257.0
			NaN <sub>3</sub> (0.9)	
Comparative Example 2		—	—	2.1

5

As shown in Table 2, in Example 2, with the combination of the sulfonic acid compound and the nitro compounds, the absorbance indicating the FV amount in the samples increased compared with the Comparative Example. Furthermore, by adding twice as much nitro compound, the absorbance increased even further. These results show that, according to Example 2, the sulfonic acid compound and the nitro compound eliminated the influence of hemoglobin in the samples.

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#### Industrial Applicability

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As described above, in the measuring method according to the present invention, by adding the sulfonic acid compound and the nitro compound to the samples, it is possible to eliminate the influence of hemoglobin in the samples as the reducing substance, thus allowing a highly-reliable measurement. Accordingly, the measuring method of the present invention can be applied to various analyses in clinical medicine, for example, and are particularly useful for measuring glycated proteins, such as glycated hemoglobin in erythrocytes, that are significant in diagnosis of diabetes.

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